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Award Number: W81XWH-06-1-0041

TITLE: Societal Interactions in Ovarian Cancer Metastases: Quorum-Sensing Hypothesis

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REPORT DATE: November 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-11-2005		2. REPORT TYPE Annual		3. DATES COVERED 1 Nov 2005 – 31 Oct 2006	
4. TITLE AND SUBTITLE Societal Interactions in Ovarian Cancer Metastases: Quorum-Sensing Hypothesis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0041	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Carrie Rinker-Schaeffer Email: crinkers@uchicago.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Chicago Chicago, IL 60422				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT NOT PROVIDED					
15. SUBJECT TERMS NOT PROVIDED					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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INTRODUCTION: It is unknown what specific biochemical and biological mechanisms control ovarian cancer metastasis. Uncovering the mechanism(s) responsible for regulating metastatic colonization in ovarian cancer requires *a fresh look from a new perspective*. The purpose of this work is to test a completely novel hypothesis: That a **Quorum Sensing** mechanism is involved in metastatic colonization. Quorum sensing is a process of cell-cell communication that bacteria use to control gene expression in response to fluctuations in cell population density. This process involves production of and response to the accumulation of a critical concentration of extracellular signal molecules called autoinducers. Quorum sensing allows bacteria to act as individuals and participate in group activities. Of relevance to metastasis is the finding that pathogenic bacteria can sense and integrate information about their numbers (quorum), physical interactions with host cells, and host-derived stress cytokines. When certain bacteria sense host vulnerability *and* have sufficient cell density, they initiate a coordinated attack by expressing virulence genes and forming organized, stable biofilms [i.e. complex, heterogeneous communities of cells within an extracellular matrix attached to a solid surface] which exacerbate disease and are refractory to a battery of therapies. This process is analogous to metastatic colonization: cells migrate toward/on target surfaces (organ-specific homing), show cell-cell and cell-matrix interactions (tumor cell-stromal cell crosstalk), remain subclinical until they can mount an effective attack (dormancy), form complex structures with channels for nutrient flow (vascularized lesions), and contain resistant cells which can cause disease recurrence (persistors). Our hypothesis predicts that groups of cancer cells can communicate through quorum sensing, initiating a program of gene expression which confers cellular changes required for efficient metastatic colonization. These cells form overt metastases, while cells that cannot form quora do not. To test this, our proposal employs a combination of experimental approaches drawn from the disciplines of experimental metastasis research and bacterial quorum sensing studies.

BODY: The proposed studies and progress toward each aspect of the work outlined in the application are described in the following sections. Both our accomplishments and challenges are described.

Aim 1. Identification of the quorum-dependent step(s) of metastatic colonization by SKOV3ip.1 cells. At the time of the submission of the proposal we had preliminary data which suggested that cells which express the MKK4 metastasis suppressor protein are less efficient at completing an early step(s) in metastatic colonization. We put forth a hypothetical model of steps of metastatic colonization are shown in Fig. 1, middle panel. We proposed that we could identify the quorum-dependent step(s) using imaging and quantitative techniques. The long term goal is to use this information to develop *in vitro* assays that model specific *in vivo* processes. Examples of *in vitro* assays which potentially could be used to model specific steps in metastatic colonization are shown Fig. 1, lower panel.

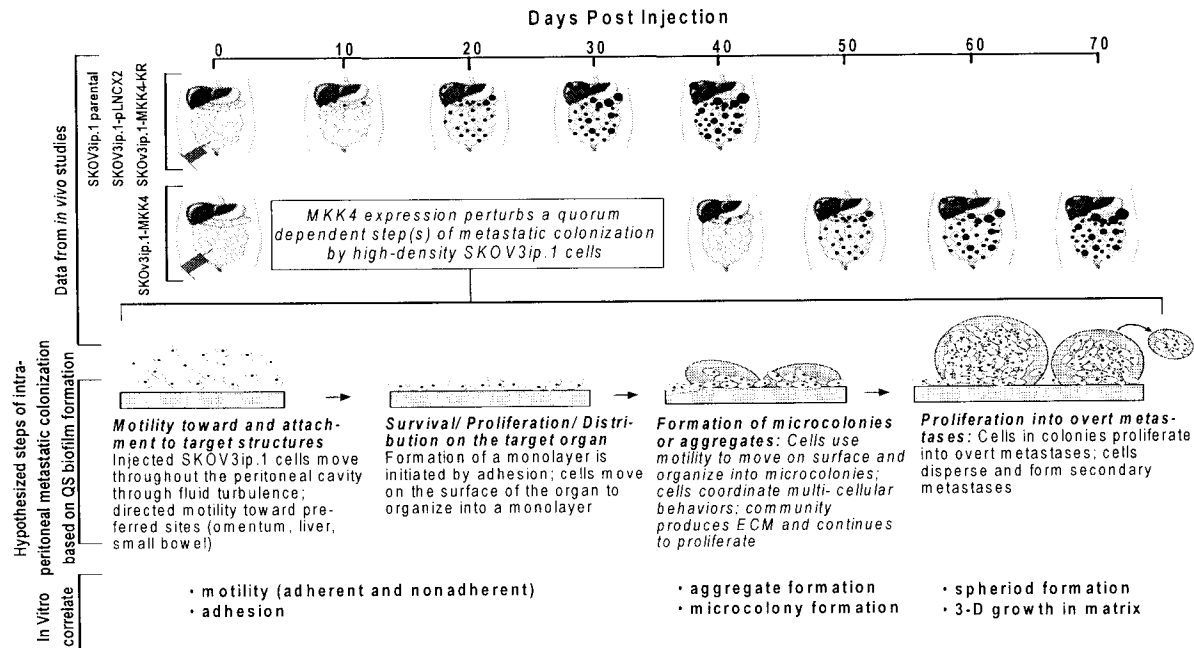


Fig. 1. Summary of data from *in vivo* studies with SKOV3ip.1 parental, SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4, and SKOV3ip.1-HA-MKK4-KR cells, as well as hypothesized steps of intraperitoneal to be assayed *in vivo* and their *in vitro* correlates. (Presented as Fig. 7 in the DOD-funded research proposal)

Hypothesis/Approach: We hypothesized that a quorum of cells grown at optimal density can proceed efficiently through steps of metastatic colonization, while suboptimal **N**, cells grown at low-density, or MKK4-expressing cells cannot. We predicted that ectopic MKK4 expression in SKOV3ip.1 cells has the same effect as reducing the **d** in the yield function $Y \sim f(N,d)t$. We posited that MKK4 affects a quorum-dependent behavior of SKOV3ip.1 cells. To test this hypothesis, we first needed to 1) establish the minimum number, or quora, of high-density SKOV3ip.1 cells necessary to form ~20 overt metastases at 20 dpi; 2) define “low-density” and 3) identify the step(s) in metastatic colonization where low-density cells persist; the quorum-dependent step. We can use these parameters to test what effect MKK4’s signaling cascade has on this process. Initial studies will use a single representative clonal cell line and data will be confirmed with additional clones to ensure statistical rigor.

Progress on Aim 1

Determining the minimum N of high-density SKOV3ip.1 cells needed for efficient MC. We have completed this study. We first had to derived a series of SKOV3ip.1 cells that express the red fluorescent protein (RFP). Cells were transfected with the appropriate expression construct, expression was confirmed in stably expressing cells, and cells with high expression of the RFP protein were selected by FACS. The *in vivo* behavior of the SKOV3ip.1-pLNCX2-RFP cells was confirmed to control for any untoward effects of the cell-tagging process. The minimum number, or quorum, of high-density [85% (4.0×10^5 cells/cm²)] SKOV3ip.1-pLNCX2-RFP cells necessary to yield ~ 20, 1-mm diameter metastases at 20 dpi will be determined empirically. The number of overt metastases formed by injection of 1×10^6 , 1×10^5 , 1×10^4 , or 1×10^3 SKOV3ip.1-

pLNCX2-RFP cells at 20 dpi was quantified. As predicted we found that there was a sharp drop-off in the Y formed by decreasing numbers of cells. Interestingly we had anticipated that this drop-off would be at 1×10^4 cells, however our study found the drop-off to be at 1×10^5 cells. As proposed additional increments of high density cells between 1×10^6 and 1×10^5 cells were used to further define the quorum. This series of studies found that the threshold quorum for cells at 85% confluence was 1×10^6 .

Determination of the rate-limiting step of metastatic colonization and the effect that MKK4 has on the quorum-dependent functions of SKOV3ip.1 cells. To determine which step in metastatic colonization is quorum-dependent we will analyze the number and distribution of cells attached to the omentum. In a pilot study (n=5) by Hickson, fluorescently-labeled SKOV3ip.1 cells could be found on the omentum, a predominant site for experimental metastases, within minutes after injection (personal communication). This localization could mimic an early step in omental caking, a well-described aspect of advanced human ovarian cancer, and further demonstrates the utility of this model. To begin these studies we focused on defining the rate-limiting steps of the experimentally determined quorum of cells at high-density (Experiment 1). We compared the SKOV3ip.1-vector only controls with the SKOV3ip.1-HA-MKK4 cells to further define the aspect of metastatic colonization affected by MKK4 activity.

Based upon our cumulative experience, we delineated this process into four general steps: 1) motility toward and attachment to target structures, 2) survival/proliferation/distribution on the target organ, 3) formation of microcolonies or aggregates, and 4) proliferation into overt metastases. Time points for evaluation of each of these parameters will be selected empirically; anticipated ranges are shown in Fig. 1. Data presented in the following subsection evaluated the effect of MKK4 on the experimentally-determined quorum's ability to attach, survive and proliferate on the target tissue. These studies indicate that MKK4's activity decreases the ability of cells to proliferate. We are currently investigating whether this is due to impaired microcolony formation.

MKK4 expression does not have a substantial effect on the number of SKOV3ip.1 cells adhering to the omentum. *In vivo* growth of SKOV3ip.1 cells recapitulates the metastatic pattern of human disease. Although MKK4 expression significantly delays the presentation of metastases, once HA-MKK4-expressing cells begin to form overt metastases, the temporal and spatial distribution of tumor burden is comparable to control cells. In order to determine whether the delay in formation of SKOV3ip.1-HA-MKK4 metastases is due to decreased adherence of injected cells, a quantitative real time PCR (qRT-PCR) assay was developed to quantitate the number of cells present on the omentum at three dpi. A standard curve was generated using genomic DNA from samples containing known numbers of SKOV3ip.1 (human) cells combined with mouse omentum homogenates, demonstrating a range of detection spanning 10^3 to 10^8 SKOV3ip.1 cells in a background of mouse genomic DNA (Fig. 2A). At three dpi, similar numbers of vector-only and HA-MKK4-expressing cells were adherent to the mouse omentum (vector mean = 5.8×10^5 cells, HA-MKK4 mean = 2.2×10^5 cells, $p = 0.06$). Although there was a modest increase in adherent vector-only cells at this time point, this difference was marginally statistically significant and was insufficient to account for

the 10-20-fold decrease in macroscopic metastases formed by HA-MKK4-expressing cells at 20 dpi.

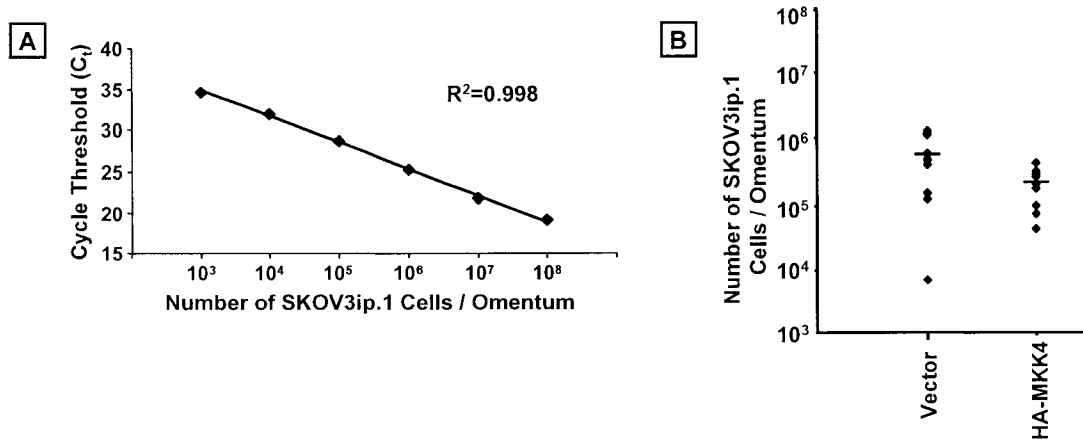


Fig. 2. HA-MKK4-expressing and vector-only SKOV3ip.1 cells initially adhere to the omentum in similar numbers. **A.** A qRT-PCR assay was designed to specifically amplify an intronic sequence in the human, but not mouse, β -globin gene. A standard curve was developed by mixing SKOV3ip.1 cell lysates with mouse omentum lysates, showing a range of detection between 1×10^3 and 1×10^8 SKOV3ip.1 cells. **B.** The number of SKOV3ip.1 cells present on the omenta from mice at 3 dpi was quantitated by the qRT-PCR assay. These data show there are nearly twice as many vector-only cells adhering to the omenta as compared to HA-MKK4-expressing cells ($p=0.06$).

Apoptosis is not increased in HA-MKK4-expressing microscopic metastases. To address the possibility that MKK4 induces apoptosis in tumor cells once they have adhered to intraperitoneal structures, histologic sections of early metastases (14 dpi) were prepared and TUNEL reaction was performed. At 14 dpi vector-only metastases are approximately 10 times larger than HA-MKK4-expressing metastases on histologic cross-section ($p<0.0004$, Fig. 3A), confirming that the MKK4-mediated growth delay is already occurring. Quantification of TUNEL-positive cells in over 25 vector-only or HA-MKK4-expressing microscopic metastases (from 7-8 animals each) revealed only rare apoptotic cells ($<1\%$) in both groups ($p = 0.43$, Fig. 3B). Histologic examination of HA-MKK4-expressing microscopic metastases at this time point revealed an inflammatory infiltrate composed of lymphocytes, histiocytes and plasma cells surrounding the tumor cells in the athymic nude mice (Fig. 3A, arrow). Although these mice have significantly decreased numbers of functional T-cells, they retain a complement of B-cells as well as increased levels of NK cells and macrophages. To address the possibility that HA-MKK4-expressing cells may preferentially invoke host immune response, leading to impaired proliferation, metastasis assays were also conducted in beige nude XID (NIH III) mice, which lack functional T, B and NK cells. By 30 dpi, beige nude XID (NIH III) mice injected with HA-MKK4-expressing cells show metastasis suppression comparable to athymic controls ($p<0.0001$ for HA-MKK4 compared to vector-only control, Fig. 3D) despite the lack of significant surrounding inflammatory infiltrate (Fig. 3C, arrow).

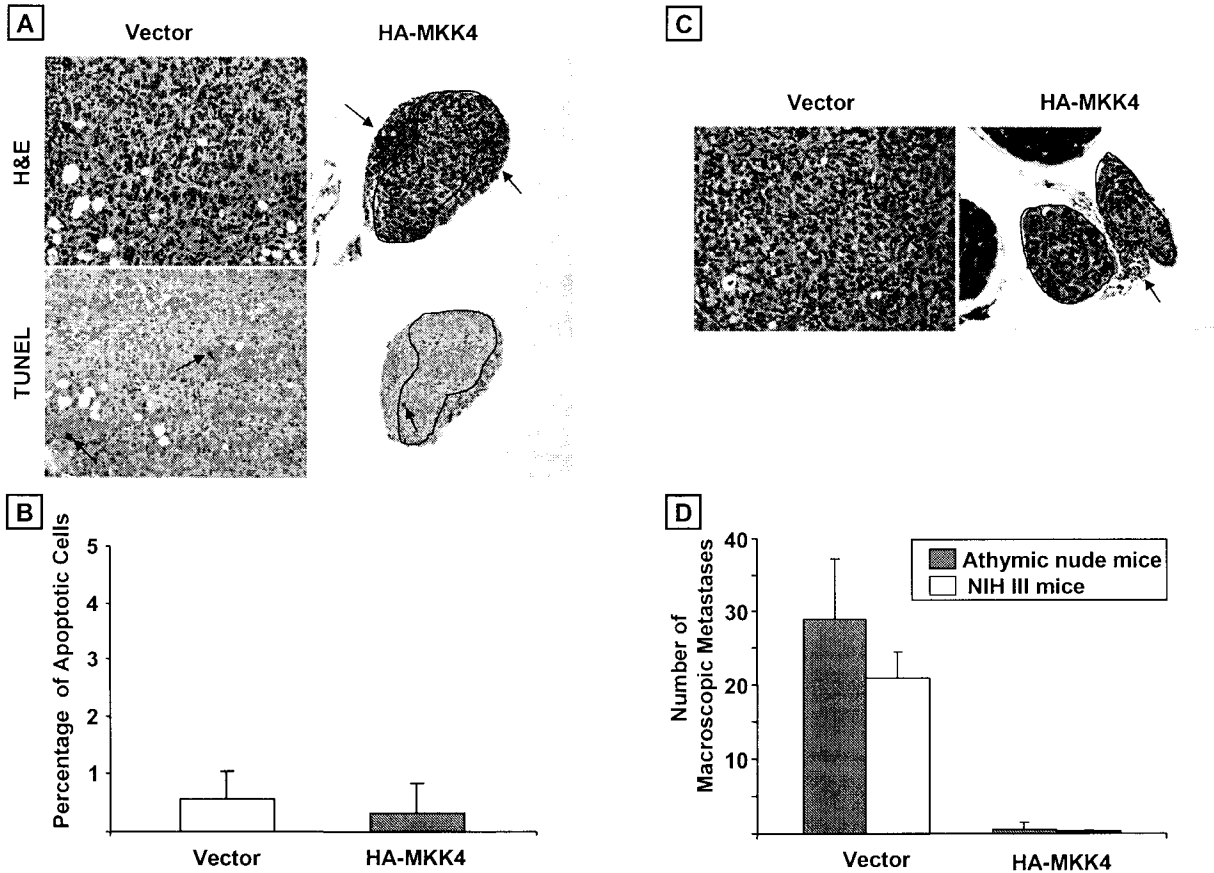


Figure 3. The *in vivo* growth delay of HA-MKK4-expressing cells cannot be attributed to increased apoptosis or surrounding host immune infiltrate. A. A quantitative histologic assessment of tissues scanned at 100x magnification showed that by 14 dpi, vector-only metastases are approximately 10 fold larger than HA-MKK4-expressing metastases ($p < 0.0004$). HA-MKK4-expressing microscopic metastases (delineated by black circles) are surrounded by an inflammatory infiltrate composed of lymphocytes, histiocytes and plasma cells in the athymic nude mice (top panel, arrow). TUNEL reaction for apoptotic cells shows only rare positive cells (bottom panel, arrows) in both groups. **B.** Quantification of TUNEL-positive cells in more than 25 vector-only or HA-MKK4-expressing metastases arising in 7-8 animals each revealed $< 1\%$ apoptotic cells in both groups ($p = 0.43$). **C.** At 14 dpi, beige nude XID (NIH III) mice injected with HA-MKK4-expressing cells showed microscopic metastases comparable in size and histology to those in athymic nude mice (delineated by black circle), but with only a scant inflammatory infiltrate (arrow). **D.** By 30 dpi, HA-MKK4-expressing cells were similarly suppressed for macroscopic metastasis formation in NIH III mice compared to athymic controls ($p < 0.0001$ for HA-MKK4 in both groups of mice compared to vector-only controls, $n = 5$ for each bar in graph).

HA-MKK4-expressing microscopic metastases show decreased proliferation. To address the possibility that MKK4-expressing cells are deficient in proliferation, SKOV3ip.1 vector-only and HA-MKK4 cells were injected into athymic nude mice and at 14 dpi animals were injected with BrdU, subsequently sacrificed and microscopic metastases were assessed for BrdU incorporation (a marker of S-phase cells) and phospho-histone H3 (pH3) expression (a marker of M-phase cells). Analysis of more than 160 microscopic metastases expressing either vector-only or HA-MKK4 revealed that BrdU incorporation was significantly decreased in HA-MKK4-expressing

metastases (Fig. 4A and B; average of 6% vs 19% positive cells, $p < 0.0001$). Similarly pH3 staining showed decreased numbers of mitotic HA-MKK4-expressing cells (Fig. 4C and D; average of 0.7% vs 2.5% positive cells, $p = 0.004$).

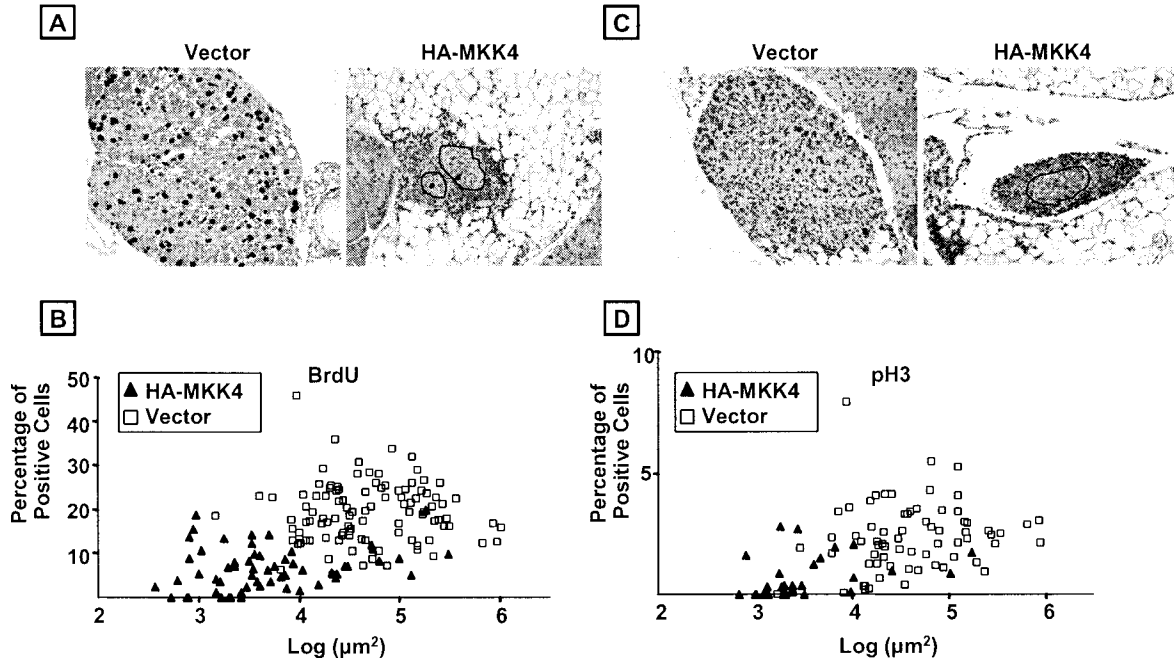


Fig. 4. HA-MKK4-expressing microscopic metastases show decreased proliferation as assessed by BrdU incorporation and pH3 staining at 14 dpi. **A.** BrdU was injected intraperitoneally 4 hours prior to the experimental endpoint. . Immunolabeling for BrdU in vector-only and HA-MKK4-expressing SKOV3ip.1 microscopic metastases at 14 dpi. **B.** More than 160 microscopic metastases were scored for size (in μm^2) and percent BrdU-positive cells using a computer aided image analysis system. Both size and BrdU incorporation were significantly decreased in HA-MKK4-expressing metastases compared to vector-only metastases ($p = 0.0003$ and $p < 0.0001$ respectively) **C.** Immunolabeling for pH3 in vector-only and HA-MKK4-expressing SKOV3ip.1 microscopic metastases at 14 dpi. **D.** More than 100 microscopic metastases were scored for size (in μm^2) and percent pH3-positive cells using a computer aided image analysis system. Both size and pH3 immunostaining for mitotic cells were significantly decreased in MKK4-expressing metastases compared to vector-only metastases ($p = 0.0008$ and $p = 0.004$ respectively).

Data from these *in vivo* studies will now enable us to prioritize both *in vivo* and *in vitro* studies to evaluate steps in metastatic colonization. For example, we now know to focus on the first 10 days of the colonization process and to specifically evaluate steps on the omental surface such as microcolony formation and induction of proliferation. Similarly, we now have a significant amount of *in vivo* biological data that will let us optimize and validate *in vitro* models thereby diminishing the possibility of studying artifacts often associated with *in vitro* models.

Development of improved approaches for *in vivo* imaging of early events in metastatic colonization. A novel aspect of the work that was proposed in our application was the use of *in vivo* imaging techniques to evaluate early steps in metastatic colonization depicted in Fig 1. Toward this goal we began studies to

determine our ability to noninvasively detect cellular events using bioluminescent and fluorescent optical imaging modalities. Using standard approaches we found that it was not possible to detect signal from bioluminescently or fluorescently labeled SKOV3ip.1-HA-MKK4 cells during the first fourteen days of the experimental metastasis assay. This was due to tissue absorbance, scattering and autofluorescence that interfered with detection of signal from a physiologically relevant number of labeled cells. We had to devise a strategy to address this unexpected technical challenge. To this end, the PI initiated a collaboration with Animal Resources Center, Optical Imaging Core Facility, and colleagues in the Department of Surgery to devise a plan to enable visualization of cells during this critically important experimental time frame.

The outcome of this collaboration was a strategy to surgically implant flexible peritoneal windows in the mice in order to circumvent scattering and absorption of transmitted light by overlying skin and peritoneum. The concept was that such windows would enable large-area, *in vivo* visualization of abdominal organs and tissues with minimal risk of infection. This would enhance both bioluminescent and fluorescent *in vivo* imaging, lowering limits of detection and eliminating tissue autofluorescence in regions of interest. Working together our team evaluated materials and devised a surgical plan that would permit unimpeded animal locomotion and have no untoward effects on animal well-being. Based upon all of the criteria and working with Dr. George Langan head of the Animal Resources center we prepared a new animal protocol for this procedure which was subsequently approved by our Institutional Animal Care and Use Committee. We have been delighted with the results of this developing technology. We have optimized window placement and the found that the windows are well-tolerated by the mice. Preliminary studies using bioluminescently labeled cells show improved ability to detect physiologically relevant number of cells attached to the omentum. Finally, our Institution has recently purchased a new Olympus OV100 microscope which will enhance our ability to detect fluorescently labeled cells. We are optimistic that the combination of the optical window technique and this new imaging modality will give us a unique ability to conduct the innovative studies proposed in our application.

Current studies

We are currently determining the minimum d of SKOV3ip.1 cells needed for efficient metastatic colonization by the quorum. We will determine the magnitude of the effect that d has on Y by the quorum. This will allow us to define high- and low-density in this model system. We will plate SKOV3ip.1-pLNCX2-RFP cells 48 hours prior to injection at a d that will result in 100% (4.6×10^5 cells/cm²), 85% (4.0×10^5 cells/cm²), 70% (3.2×10^5 cells/cm²), 50% (2.3×10^5 cells/cm²), and 20% (0.9×10^5 cells/cm²) confluence at the time of injection. We predict that there will be a sharp drop-off in Y formed by cells at $d \leq 70\%$ confluence. We are also beginning the imaging studies aimed at evaluating early steps of metastatic colonization depicted in Fig. 1.

Aim 2. To test whether SKOV3ip.1 cells produce and respond to quorum sensing autoinducers. The rationale for these studies is that bacterial quorum sensing compounds have routinely been identified by preparing conditioned media from high-density bacterial cells, transferring it to bacterial cells at low-density and monitoring the low-density bacterial cells for artificial activation of high-density phenotype. The ability to

induce the expression of high-density behaviors in low-density cells using this scheme is the hallmark study of quorum sensing and suggests that an compound or autoinducer is secreted into the extracellular environment. Once established, these assays are routinely used as the readout for auto inducer activity in purification and identification schemes for quorum sensing signal molecules.

Hypothesis/Approach: We hypothesize that conditioned from high-density SKOV3.ip1 cells will induce quorum sensing behavior(s) in low-density cells. Initially, we planned to use *in vivo* metastasis assays to test the effects of treatment with conditioned media from high-density cells.

Progress on Aim 2

The first step toward this is to determine the effect of cell density on parameters of metastatic colonization. We can use the data generated in Aim 1 to conduct these studies. We are currently testing the effect of density on metastatic colonization. Once this is completed we can move onto the treatments with the high-density conditioned media.

Aim 3. To test known quorum sensing signaling molecules for new functions in the induction of quorum sensing-like behavior. Rationale: It is possible that bacterial and cancer cell-produced quorum sensing auto inducers have similar chemical structures. We will test this possibility by assaying the phenotypic responses of SKOV3ip.1 cells to bacterial autoinducers and the responses of bacteria to putative QS molecules present in condition media prepared from SKOV3ip.1 cells.

Hypothesis/Approach: There is growing evidence that prokaryotes and eukaryotes have conserved cell-counting and signaling mechanisms. Further, eukaryotes possess quorum-quenching capabilities. We hypothesize that bacterial and cancer cell-produced quorum sensing auto inducers have similar chemical structures.

Progress on Aim 3

We currently have assays for the detection of AI-1 and AI-2 up and running in our laboratory. We have to first demonstrate biological activity of the conditioned media work proposed in Aim 2 in order to identify appropriate conditions and time points for the assays. We also have to identify appropriate density dependent behaviors in cells before we attempt studies to test the ability of known quorum sensing signaling molecules to promote metastatic colonization in SKOV3ip.1 cells at low density.

KEY RESEARCH ACCOMPLISHMENTS:

Development and characterization of fluorescently- and bioluminescently-tagged SKOV3ip.1-vector and SKOV3ip.1-HA-MKK4 cells for *in vivo* studies.

Determination of the minimum number (**N**) of high-density cells for efficient metastatic colonization.

Determination that MKK4 activity does not significantly effect: 1) the number of SKOV3ip.1 cells that attach to the omentum or 2) the percentage of attached cells which under go apoptosis.

Identification of role for MKK4 activity in inhibits proliferation of SKOV3ip.1 cells attached to the omentum during the first 14 days post injection. This indicates that quorum-dependent behaviors of the SKOV3ip.1 cells which are perturbed by MKK4 activity occur within the first 14 days of metastatic colonization.

Developed an alternative (optical windows) strategy of the fluorescent imaging of SKOV3ip.1 cells during peritoneal colonization. This should circumvent significant problems caused by tissue absorbance, autofluorescence and scattering.

REPORTABLE OUTCOMES: We are currently preparing manuscripts reporting the biological mechanism of MKK4-mediated suppression of colonization and the surgical technique for window implantation.

CONCLUSION: Thus far the work is going according to the experimental plan put forth in the funded research proposal. Some of the work, such as the optical imaging studies, was trickier than anticipated. We are delighted that we were able to develop a new technique to address this problem. We are currently pursuing the remainder of the work as described.

REFERENCES: None Required

APPENDICES: None, all work presented in the narrative.

SUPPORTING DATA: Provided in the narrative.